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Characterization of Elk, a Brain-Specific Receptor Tyrosine Kinase

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The *elk* gene encodes a novel receptorlike protein-tyrosine kinase, which belongs to the *eph* subfamily. We have previously identified a partial cDNA encompassing the *elk* catalytic domain (K. Letwin, S.-P. Yee, and T. Pawson, Oncogene 3:621–678, 1988). Using this cDNA as a probe, we have isolated cDNAs spanning the entire rat *elk* coding sequence. The predicted Elk protein contains all the hallmarks of a receptor tyrosine kinase, including an N-terminal signal sequence, a cysteine-rich extracellular domain, a membrane-spanning segment, a cytoplasmic tyrosine kinase domain, and a C-terminal tail. In both amino acid sequence and overall structure, Elk is most similar to the Eph and Eck protein-tyrosine kinases, suggesting that the *eph*, *elk*, and *eck* genes encode members of a new subfamily of receptorlike tyrosine kinases. Among rat tissues, *elk* expression appears restricted to brain and testes, with the brain having higher levels of both *elk* RNA and protein. Elk protein immunoprecipitated from a rat brain lysate becomes phosphorylated on tyrosine in an in vitro kinase reaction, consistent with the prediction that the mammalian *elk* gene encodes a tyrosine kinase capable of autophosphorylation. The characteristics of the Elk tyrosine kinase suggest that it may be involved in cell-cell interactions in the nervous system.

A frequently used mechanism by which the cells of metazoan organisms communicate involves the binding of growth factors to transmembrane receptors with protein-tyrosine kinase activity. In vertebrates, receptor tyrosine kinases and their ligands participate in embryonic development, differentiation of specific cell lineages, biological activities of mature cells, cellular proliferation, and regulation of cell metabolism (17). All receptorlike tyrosine kinases have a similar structural organization, with an extracellular ligand-binding domain, a membrane-spanning segment, and a cytoplasmic enzymatic domain. However, a more detailed comparison allows the division of receptor tyrosine kinases into subfamilies whose members are particularly closely related to each other. Prototypes for these subfamilies include the epidermal growth factor receptor, the β-type platelet-derived growth factor receptor (β-PDGFR), and the insulin receptor (reviewed in references 22 and 27). It seems likely that the members of each subfamily have evolved by a process of gene duplication to accommodate the complex cell-cell interactions of higher organisms. Consistent with this notion, the genes for β-PDGFR and the macrophage colony-stimulating factor receptor are located in a tandem array on human chromosome 5 (19), while the α-PDGFR and *c-kit* genes are similarly linked on chromosome 4 (1).

The *eph* gene encodes a transmembrane protein-tyrosine kinase that differs from previously identified receptors primarily in the structure of its extracellular domain, which contains a single cysteine-rich region (9). The *eph* enzymatic domain also appears distinct from that of other subclasses of transmembrane tyrosine kinases. The suggestion that *eph* might be the prototype for a novel subfamily of receptorlike tyrosine kinases has received support from the isolation of a cDNA for the *elk* tyrosine kinase (12). The initial partial *elk* cDNA, as isolated from a rat brain expression library, encodes the catalytic domain and C-terminal tail of a presumptive kinase which is closely related to *eph*. Recently, isolation of the full-length cDNA encoding an additional member of this subfamily, designated *eck*, has been reported (13).

Here, we describe the isolation and sequencing of cDNAs covering the whole *elk* coding region and define the patterns of expression and kinase activity of the *elk* gene product.

MATERIALS AND METHODS

Isolation of cDNA clones. cDNA expression libraries (λgt11 rat brain cDNA library [Clontech Laboratories; RL 1002b] and λZAP rat cerebellum cDNA library [gift from J. Boulter]) were screened by established procedures (14). Phage (5×10^5 to 7.5×10^5 PFU) were mixed with 1.5 ml of an overnight culture of *Escherichia coli* LE392 or BB4, incubated 15 min at room temperature, and plated onto LB agar at a density of 5×10^4 PFU/150-mm dish. Plaques were allowed to develop for 6 to 8 h, and duplicate lifts were taken on nitrocellulose filters (Schleicher & Schuell) and hybridized overnight with a ^{32}P-labeled DNA probe. Filters were washed twice for 30 min in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 65°C and exposed to Kodak XRP film.

Subcloning and determination of nucleotide sequence. DNA was prepared from plaque-purified λgt11 bacteriophage following the procedure of Silhavy et al. (21), and cDNA inserts were transferred into a phagemid, pGEM7Zf(+) (Promega Biotec). pBluescript SK phagemids, resident in λZAP bacteriophage, were rescued by cocultivation with helper phage M13K07, as suggested by the manufacturer (Stratagene Cloning Systems). Nested deletions covering cDNA inserts were generated as described by Henikoff (7). Single-stranded DNA was prepared by superinfection with phage M13K07 (23), and the nucleotide sequence was determined by the dideoxynucleotide chain termination method (20). Both strands were sequenced. Sequence analysis was assisted by GCG computer programs (5a) and by a program, *maligned*, kindly provided by S. Clark (4).

RNase protection analysis. RNA was purified by an acid

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guanidinium thiocyanate-phenol-chloroform extraction method (3). Samples of total RNA (5 μg) were subjected to RNase protection analysis (8). ^{32}P-labeled antisense RNase protection probes were synthesized with T_7RNA polymerase by using the *Hin*dIII-linearized p*elk*RHB-8 plasmid as a template. p*elk*RHB-8 was generated from a 147-nucleotide *Bst*XI-to-*Hin*cII fragment that spans rat *elk* nucleotides 2991 to 3138 that was cloned into pGEM-3Z (Promega Biotec) between the unique *Xba*I and *Hin*cII sites.

Preparation of antibodies. A *Pvu*II fragment spanning nucleotides 2918 to 3188 of the full-length *elk* cDNA, which encodes 90 amino acids at the carboxy terminus of the putative *elk* product (amino acids 852 to 941), was subcloned into a pATH bacterial expression vector (26) and expressed in *E. coli*. The TrpE-*elk* fusion protein was purified by preparative SDS-polyacrylamide gel electrophoresis and used to immunize two New Zealand White rabbits.

Preparation of β-galactosidase–Elk lysogen. A λB1 lysogen able to express a β-galactosidase–Elk fusion protein was made by infecting *E. coli* Y1089 with the λB1 phage and screening for colonies capable of growing at 32°C but not at 42°C. Whole-cell lysates were prepared by growing the lysogenic bacteria at 32°C until they reached an optical density at 550 nm of approximately 0.5. The temperature was shifted to 42°C for 15 min, isopropyl-β-D-thiogalactopyranoside was added to 1 mM, and the culture was incubated for 60 min at 37°C. Cells were then pelleted by centrifugation, suspended in cracking buffer (10 mM sodium phosphate [pH 7.2], 1% [vol/vol] β-mercaptoethanol, 1% [vol/wt] SDS, 6 M urea) diluted with an equal volume of 2× SDS sample buffer, and boiled for 5 min. Lysates were fractionated on a 7.5% SDS-polyacrylamide gel and immunoblotted with anti-β-galactosidase antibodies (Sigma) or antiphosphotyrosine antibodies.

Immunoprecipitation. Approximately 0.1 g of rat tissue (6- to 8-week-old Fisher rats) was homogenized in 1 ml of lysis buffer (150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, 100 μM sodium orthovanadate, 100 μM NaF, 10 μg of aprotinin per ml, 10 μg of leupeptide per ml, 1 μM phenylmethylsulfonyl fluoride), incubated on ice for 10 min, and clarified by centrifugation at 12,000 × *g* for 30 min at 4°C. The lysate was then either used directly or flash-frozen in ethanol-dry ice and stored at −70°C. Lysate (300 μl) was incubated with 5 μl of rabbit anti-elk serum and 100 μl of 10% protein A-Sepharose (Pharmacia-LKB Biotechnology) for 2 to 3 h. The immune complexes were washed four times in lysis buffer, boiled for 2 min in 50 μl of SDS sample buffer, and fractionated on a 7.5% SDS-polyacrylamide gel. Separated proteins were transferred to an Immobilon-polyvinylidene difluoride membrane (Millipore Corp.) and probed with rabbit anti-elk serum (diluted 1:100) for 90 min. Bound antibody was then detected by an anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma), with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as substrates.

Immune complex kinase assays. Washed immunoprecipitates were suspended in 30 μl of 20 mM Tris-HCl (pH 7.5)–20 mM $MnCl_2$–10 μCi of [γ-^{32}P]ATP (3,000Ci/mmol; Amersham) and incubated at 37°C for 10 min. Reaction mixtures were mixed with an equal volume of 2× SDS loading buffer, boiled for 2 min, and fractionated on a 7.5% SDS-polyacrylamide gel. The dried gel was exposed to Kodak XRP film. Subsequently, to enrich for phosphotyrosine, the gel was treated with 1 M KOH for 90 min at 55°C, dried, and again exposed to Kodak XRP film.

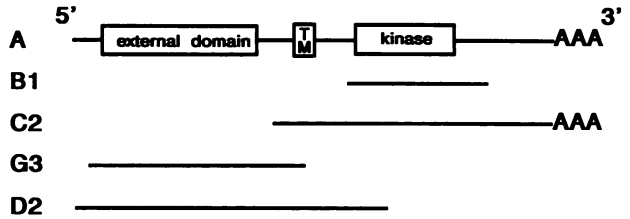


FIG. 1. Diagrammatic representation of *elk* cDNAs. A, Arrangement of functional domains in predicted translation product of the full-length *elk* cDNA; B1, previously described partial *elk* cDNA (12) used as a DNA probe for subsequent screening; C2, *elk* cDNA isolated from λgt11 rat library; G3 and D2, *elk* cDNAs isolated from λZAP rat cerebellar library; TM, transmembrane domain.

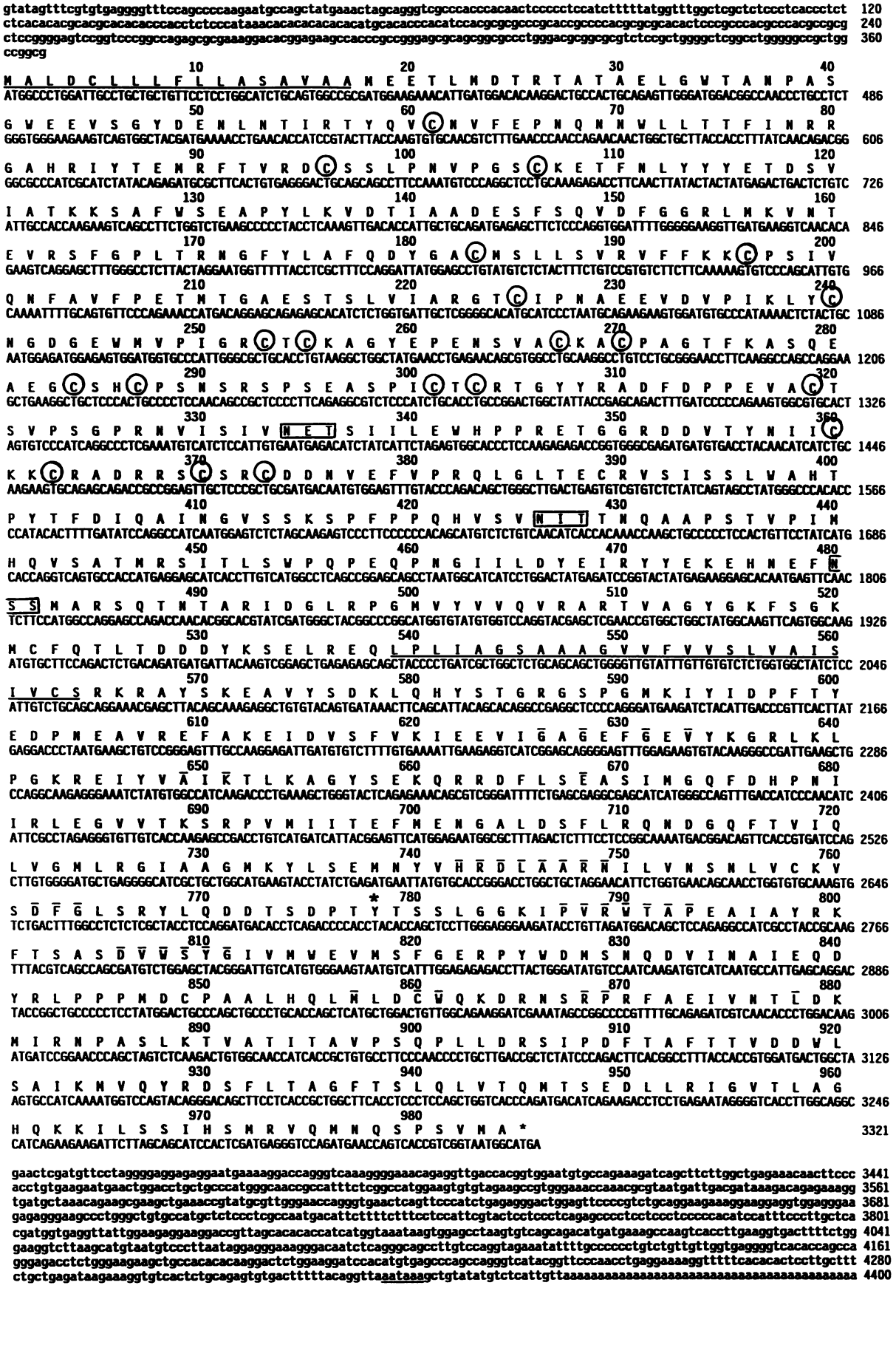
Phosphoamino acid analysis. Phosphoamino acids were analyzed as described previously (5). Products of the kinase reaction were separated on a 7.5% SDS-polyacrylamide gel and transferred to an Immobilon membrane, and the band of interest was hydrolyzed in 5.7 M HCl at 110°C for 90 min. The products of acid hydrolysis were resolved by two-dimensional electrophoresis on a thin-layer cellulose plate (Merck) at pH 1.9 and pH 3.5.

Nucleotide sequence accession number. The nucleotide sequence of *elk* cDNA has been submitted to GenBank under the accession number M59814.

RESULTS

Isolation and analysis of *elk* cDNA. In an effort to obtain rat cDNA clones spanning the complete coding region for the *elk* protein-tyrosine kinase, we probed 5 × 10^5 PFU of a random oligonucleotide-primed λgt11 rat brain cDNA library with the previously described (12) 1.5-kbp *elk* cDNA. Among the five positive clones isolated, the one with the largest insert contained 2.8 kbp of the *elk* cDNA, of which ~1.1 kbp covered the whole 3′ untranslated region and the remaining ~1.7 kb was derived from the coding region of the cDNA. To obtain sequences derived from the more 5′ end of the *elk* cDNA, 7.5 × 10^5 phage of a λZAP rat cerebellar cDNA library were screened. The screening yielded 11 independent isolates, at least 2 of which contained an additional ~1.6-kbp sequence derived from the 5′ end of the *elk* cDNA (Fig. 1).

A composite nucleotide sequence of the *elk* cDNA, as compiled from the three overlapping isolates, spans 4,360 bp (Fig. 2). The cDNA sequence contains a single open reading frame of 2,955 bp which is preceded by 367 bp of a highly repetitive 5′ untranslated region and followed by 1,088 bp of a 3′ untranslated region. The 3′ untranslated region includes the consensus polyadenylation signal AATAAA as well as a portion of the poly(A) tail. The 5′ untranslated region is CT rich and contains several in-frame stop codons. The *elk* coding region likely begins at nucleotide 367; the ensuing codon is the first in-frame methionine, and the sequence of the surrounding region is in good agreement with the consensus sequence for a translation initiation site (10). The predicted product of the *elk* cDNA contains 984 amino acid residues, giving it a relative molecular weight of approximately 110,000, and bears all the structural hallmarks of a typical tyrosine kinase receptor. Its calculated hydrophobicity profile predicts the existence of two highly hydrophobic regions (data not shown); the first region resides at the extreme NH_2 terminus, and its amino acid composition indicates that it may represent a cleavable signal peptide



(24). The second hydrophobic region, located approximately in the middle of the molecule, contains 23 nonpolar residues and is immediately followed by the highly basic Arg-Lys-Arg motif, which suggests that it may serve as a transmembrane anchor (25). The amino-terminal two-thirds of the putative extracellular region contain 20 cysteine residues clustered into a single cysteine-rich domain; closer to the transmembrane segment, three potential N-linked glycosylation sites have been identified. Most of the cytoplasmic region is occupied by an uninterrupted enzymatic domain, which contains all the sequence motifs considered diagnostic of protein-tyrosine kinases (6); the last ~100 residues form a receptor-specific carboxy-terminal tail.

In overall structure, the predicted *elk* product is very similar to two other receptorlike tyrosine kinases, *eph* and *eck* (Fig. 3). The products of all three genes are roughly the same size (*elk*, 984 residues; *eph*, 984 residues; *eck*, 974 residues). Although the genes differ slightly in the length of their signal peptides, cleavage of the signal peptide could in each case yield a processed, mature form that begins with the same conserved N terminus. Similarity between the extracellular domains of Elk, Eph, and Eck exceeds 40%. There are several islands of conserved amino acid residues dispersed over the extracellular domains of all three putative receptors; the most striking feature is the complete conservation of both the number and the location of the cysteine residues in their cysteine-rich regions. Though the least conserved sequences are those flanking the transmembrane domain, the location of the membrane-spanning segment is well preserved in all three receptors. The similarity between Elk, Eph, and Eck is highest throughout the kinase domain (>60%) and decreases only marginally over the remaining C-terminal tail (>40% similarity). The Elk, Eph, and Eck kinase domains are much more closely related to one another than to the kinase domains of any other tyrosine kinase.

Expression of *elk* is limited to rat brain and testes. We have previously analyzed the expression of rat *elk* mRNA by Northern (RNA) blotting, which revealed a 4.0-kb transcript in RNA extracted from rat brain and a much weaker signal of the same mobility in RNA from testes. Since even the signal from brain RNA was weak, we decided to reexamine *elk* expression by a more-sensitive RNAse protection assay. We analyzed a variety of tissues from 6-week-old Fisher rats for *elk* RNA and detected the appropriate RNAse-resistant *elk* fragment only in RNA isolated from brain and testes (data not shown).

To investigate the expression of the Elk protein, we immunized rabbits with a bacterially synthesized TrpE-Elk fusion protein. In this antigen, a 37,000-molecular-weight N-terminal TrpE moiety is followed by a 10,000-molecular weight fragment derived from the extreme C terminus of Elk. On Western blots (immunoblots), the antibody recognized a single band of M_r ~130,000 in lysates from brain and testes but not from any other rat tissue investigated (ovary, heart, lung, kidney, spleen, thymus, salivary glands, striated muscle, skin, small intestine, large intestine, and liver) (Fig. 4A; other data not shown). The difference between the calculated size of 110,000 and the observed mobility of ~130,000 is probably caused by the addition of carbohydrate and gives the rat Elk protein an apparent molecular weight similar to those of human Eph and Eck.

Elk has intrinsic tyrosine kinase activity. The initial isolation of an *elk* cDNA employed a screen with antiphosphotyrosine antibodies (12), implying that *elk* encodes a protein with an intrinsic tyrosine kinase activity. To test the activity of the *elk* putative enzymatic domain in bacteria, we established the original *elk* λgt11 bacteriophage (B1, Fig. 1) as an *E. coli* lysogen. Bacterial extracts were analyzed by Western blotting with antiphosphotyrosine antibodies. While no immunoreactive bands were detected prior to induction of the *elk* lysogen, numerous polypeptides were recognized by antiphosphotyrosine antibodies after expression of the cDNA encoding the *elk* cytoplasmic domain (Fig. 5), suggesting that the *elk* cytoplasmic region can act as a protein-tyrosine kinase in bacteria.

Subsequently, we tested rat Elk for its ability to autophosphorylate in an immune complex kinase reaction. Anti-Elk immunoprecipitates were prepared from different rat tissues and then incubated in the presence of [γ-^{32}P]ATP. A phosphorylated protein with a relative mobility of ~130,000 was detected only in reactions containing immunoprecipitates from brain and, to a lesser extent, from testes (Fig. 4B). This ^{32}P-labeled protein comigrated with Elk identified by Western blotting and is therefore in all likelihood the Elk protein itself. The persistence of the ^{32}P signal through the treatment with 1 M KOH indicated that phosphorylation was mainly on tyrosine residues. Phosphoamino acid analysis showed directly that the in vitro-phosphorylated 130,000-molecular-weight protein in anti-Elk immunoprecipitates contained phosphotyrosine (Fig. 4C). Taken together, these results show that the Elk protein has an intrinsic protein-tyrosine kinase activity and is capable of autophosphorylation in vitro. The site(s) of Elk phosphorylation is not yet identified, though tyrosine 788, within the kinase domain of Elk, is a candidate, being in a position analogous to the autophosphorylation sites of several other protein-tyrosine kinases (Fig. 2). Curiously, in Elk this tyrosine is flanked by threonine and serine residues.

DISCUSSION

We have deduced the primary structure of the Elk protein-tyrosine kinase and investigated its patterns of expression and protein kinase activity. The cDNA sequence, as compiled from several phage isolates, spans 4,360 bp and must therefore represent virtually the entire *elk* mRNA, which has been previously estimated (12) at approximately 4 kb. We believe that this cDNA sequence contains the whole coding region of *elk* since (i) the proposed initiation codon is embedded in an environment favorable for the initiation of translation (10) and is preceded in-frame by at least four termination codons; (ii) the region immediately following this presumptive initiation methionine is highly hydrophobic

FIG. 2. Nucleotide and amino acid sequences of the *elk* cDNA and its putative translation product. Nucleotides are numbered on the right. Amino acids are numbered above the sequence, starting with the presumed initiating methionine. The putative signal peptide and transmembrane regions are underlined, as is the consensus polyadenylation sequence aataaa in the 3′ untranslated region. Broken lines above the amino acid sequence identify regions conserved among protein kinases. Tyrosine 788, which corresponds to a frequent site of tyrosine kinase autophosphorylation, is marked by an asterisk. Conserved cysteine residues in the ectodomain are circled, and predicted N-glycosylation sites are boxed.

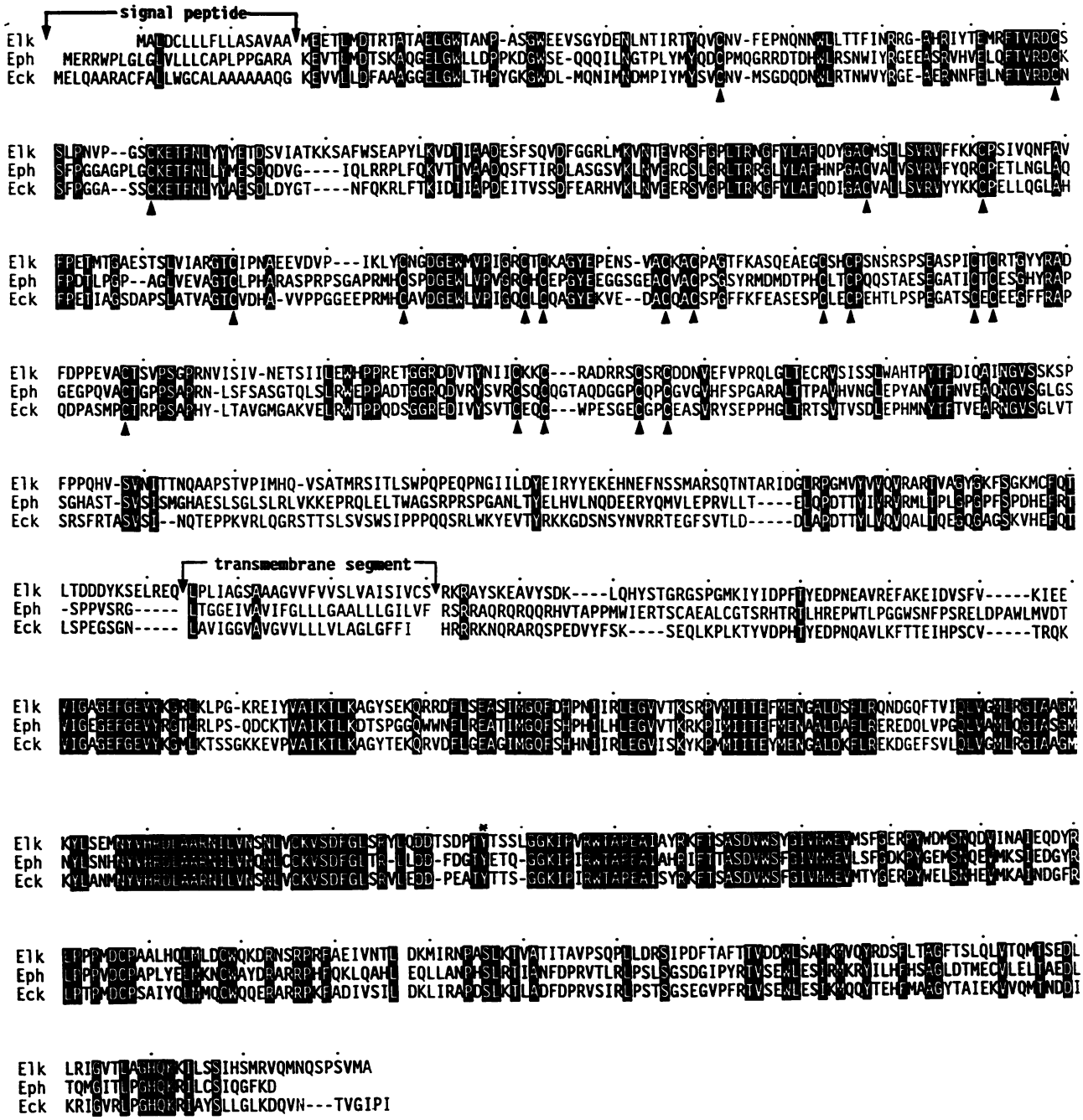


FIG. 3. Comparison of amino acid sequences of Elk, Eph, and Eck. Amino acid residues conserved among all three proteins are shown in white; conserved cysteines in the ectodomain are marked by black triangles. An asterisk indicates the position of a possible autophosphorylation site, at tyrosine 788.

and conforms well to characteristics of a cleavable signal peptide (24); and (iii) the size and overall structure of the predicted Elk protein are very similar to those of two other related protein-tyrosine kinases, namely Eph and Eck.

On the basis of the similarity between the Elk and Eph kinase domains, Elk was previously recognized as a member of a distinct family of receptorlike tyrosine kinases, of which Eph is considered the prototype. In addition to the three members whose full-length cDNAs have been isolated so far (*eph*, *eck*, and *elk*), the *eph* subfamily probably contains additional members, several of which are closely related to *elk* and are also expressed in the nervous system. Southern analysis performed at high stringency on rat genomic DNA suggested that the *elk* gene is present as a single copy in the rat genome. However, under slightly relaxed hybridization conditions, the *elk* probe readily cross-hybridized with a variety of genomic restriction fragments, indicating the existence of several highly related genes (unpublished re-

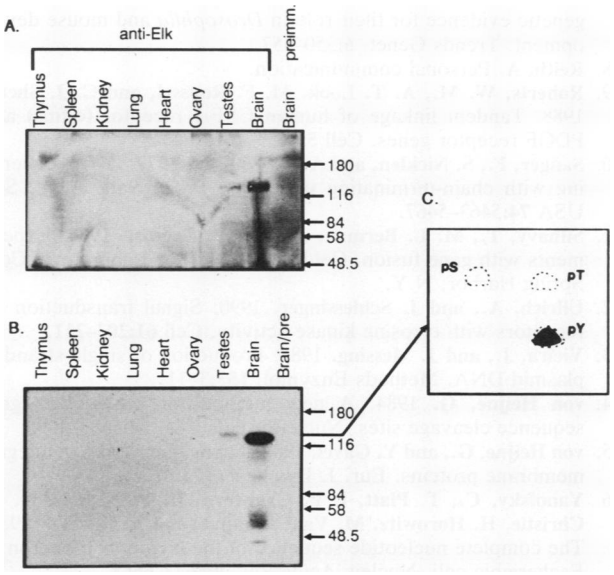


FIG. 4. Expression and autophosphorylating activity of Elk from different rat tissues. A ~0.1-g portion of the indicated tissues from a 6-week-old rat was homogenized and suspended in 1 ml of kinase lysis buffer as described in Materials and Methods. A 300-μl portion of the lysate was incubated with 10 μl of anti-Elk rabbit serum for 90 min at 4°C. (A) To analyze Elk expression, immunoprecipitates were resolved on a 7.5% SDS-polyacrylamide gel and immunoblotted with anti-Elk serum. (B) To analyze the Elk autokinase activity, immunoprecipitates were incubated at 37°C for 15 min in 30 μl of kinase reaction buffer containing 1 μCi of [γ-^{32}P]ATP (0.33 nM). Kinase reaction products were fractionated on a 7.5% SDS-polyacrylamide gel and detected by autoradiography for 6 h. (C) Products of an in vitro kinase reaction performed on an anti-Elk immunoprecipitate from rat brain were fractionated on a 7.5% SDS-polyacrylamide gel and transferred to an Immobilon membrane. The portion of the membrane containing the Elk protein was exposed to 5.7 M HCl at 110°C for 60 min. The products of hydrolysis were mixed with unlabeled phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) and separated by two-dimensional electrophoresis on a thin-layer cellulose plate. Following autoradiography, the positions of the pS, pT, and pY markers were visualized with ninhydrin. Numbers on the right in panels A and B are molecular weights in thousands.

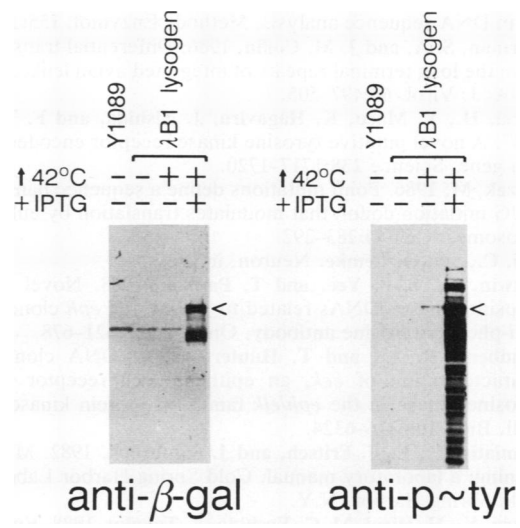


FIG. 5. A β-galactosidase–Elk fusion protein expressed in bacteria autophosphorylates and phosphorylates bacterial proteins on tyrosine in vivo. The β-galactosidase–Elk fusion protein was induced by incubating growing cultures of a λB1 lysogen briefly at 42°C and then adding isopropyl-β-D-thiogalactopyranoside (IPTG). Lysates prepared from uninfected Y1089 cells and λB1 lysogenic cells, both induced and uninduced, were fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with either anti-β-galactosidase antibodies (anti-β-gal) or antiphoshotyrosine (anti-P-tyr) antibodies. The β-galactosidase–Elk polypeptide is indicated with arrowheads.

sults). Indeed, a number of partial cDNAs for tyrosine kinases closely related to *elk* have recently been isolated (2, 11, 16, 18).

Comparison of the *eph*, *eck*, and *elk* cDNAs reveals a remarkable degree of amino acid conservation among their translation products (Fig. 3). In their processed forms, they all appear to share the same N terminus; in their respective extracellular regions, they contain a single cysteine-rich domain, in which all the cysteine residues are invariant. They share more than 60% identity in their enzymatic domains, and even their extreme C termini are more than 40% similar. Assuming that the extracellular domains of Elk, Eph, and Eck bind soluble or cell-surface associated ligands, the high degree of similarity in this region suggests that their ligands may also be closely related.

In contrast to this high level of structural similarity, the patterns of expression of these three related genes are quite different. *eph* expression has been found in several tissues, including lung, liver, kidney, and testes (15), and *eck* expression is highest in lung, skin, ovary, small intestine, and generally in tissues derived from epithelial cells (13). Expression of *elk* is much more restricted, being detected predominantly in the brain and at a low level in testes. We are presently investigating the expression of Elk and related kinases during mouse embryonic development to determine the cell types in which they may function. We anticipate that Elk plays a role in cell-cell interactions in the central nervous system.

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